Microbiological Research 162 (2007) 308–321

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Microbiological Research
www.elsevier.de/micres

Diversity of the fatty acids of the *Nostoc* species and their statistical analysis

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Accepted 17 January 2006

KEYWORDS
Nostoc;
Cyanobacteria;
Fatty acids;
GC-MS;
Statistic analysis

Summary

Low molecular, hydroxy, dioic, saturated and unsaturated fatty acids were determined of six cyanobacterial species belonging to genus *Nostoc* and in different habitats: freshwater, terrestrial, and as well as symbionts. There are large variations in individual fatty acid contents according to species, and location of the genus *Nostoc*. Statistical analysis of variability of fatty acids belonging to the genus *Nostoc* is reported.

Introduction

Nostoc, a genus of filamentous, heterocystous, cyanobacteria, is widely distributed in the free-living state (Dodds et al., 1995; Potts, 2000). It is also the most common phycobiont in N_2-fixing lichens and occurs as the N_2-fixing symbiont in a small and diverse group of green plants, and about 10% of lichens have cyanobacteria (e.g., *Nostoc*) as the main or only photosynthetic partner (Rai et al., 2002). The cyanobacterium *Nostoc* has been used as a source of protein, vitamin and unsaturated fatty acids for humans and animals (Gao, 1998; Takenaka et al., 1998; Liu et al., 2005). The medicinal value of cyanobacteria was appreciated as early as 1500 BC, when strains of *Nostoc* were used to treat gout, fistula and several forms of cancer (Pietra, 1990). The very high incidence of novel, biologically active compounds isolated so far indicates that cyanobacteria are a rich source of potentially useful natural products (Moore, 1996; Li and Liu, 2003; Han et al.,

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doi:10.1016/j.micres.2006.01.010

2004). A different Nostocales species, the majority of which are *Nostoc* and *Anabaena* species produce over 200 secondary metabolites (Dembitsky and Rezanka, 2005), including fatty acids, having activities such as anti-HIV, anticancer, antifungal, antimalarial and antimicrobial (Burja et al., 2001; Donia and Hamann, 2003). Many microalgae and cyanobacteria are a valuable source of a wide spectrum of lipids with different potential applications (Liu et al., 2005). Especially interesting are the polyunsaturated fatty acids (PUFA), including the essential fatty acids linoleic acid, α-linolenic acid, γ-linolenic acid, octadecatetranoic acid, and eicosapolyenoic acids (Vargas et al., 1998; Wang et al., 2000). Essential fatty acids are precursors of prostaglandins and, as such, are becoming increasingly important in the pharmaceutical industry (Becker, 1994). Differences in the fatty acid composition of cyanobacteria led Kenyon and co-workers (Kenyon, 1972; Kenyon et al., 1972) to suggest criteria for their classification into four groups: the first group includes cyanobacterial strains devoid of PUFA, containing only saturated and mono-unsaturated fatty acids. The second and third groups consist of strains containing either α-linolenic acid (ALA, 18:3ω3), or γ-linolenic acid (GLA, 18:3ω6), respectively, while strains belonging to group four also contain octadecatetraenoic acid (18:4ω3). These findings were later confirmed by Murata et al. (1992). Lipids, fatty acids, and also other compounds provide interesting information for assignment of the taxonomic position and some correlations with morphological properties of cyanobacteria (Vasyurenko and Frolov, 1986; Kaneda, 1991; Romano et al., 2000; Li and Watanabe, 2001; Dembitsky and Rezanka, 2005).

In this paper we describe the application of serially coupled capillary columns with consecutive nonpolar and semipolar stationary phase coating for separation of low molecular, hydroxyl, dioic, saturated and unsaturated fatty acids (Dembitsky et al., 1999; Rezanka et al., 2003) isolated from six strains of filamentous, heterocystous, nitrogen-fixing cyanobacteria *Nostoc* species, which were isolated from natural environments. The results obtained are discussed, together with literature data, to verify previously proposed fatty acid distributions for cyanobacteria belonging to the genus *Nostoc*.

Here we described the application of cluster analysis for the identification of fatty acids from different members of genus *Nostoc* by means of commercial statistical package STATISTICA. This software system was very useful for the further application of the results hence we used it for representation of the chemotaxonomic relation and also for interpretation of the fatty acids contents from the cyanobacteria of genus *Nostoc*.

Materials and methods

Cyanobacterial strains and culture conditions

Six strains were used in the present study. Among these strains, *Nostoc commune* Vauch., originally was found in soil at Negev Desert. *Nostoc paludosum* Kütz was found in rock at Dead Sea area. The freshwater species of *Nostoc pruniforme* Ag. and *Nostoc verrucosum* Vauch. were isolated from Lake of Kinneret and Lake Hula, respectively. Two symbiotic *Nostoc* species were isolated as photobiont from *Collema cristatum* and *Peltigera hydrothyria*, respectively. All the strains were cultivated in BG-11 medium as described by us previously (Rezanka et al., 2003; Torres et al., 2003). The cultures were grown in 1 L of medium in 2-L flasks equipped with aeration by a filter with 0.25-mm pore size. All cultures were sparged with sterile air and maintained at 22–27 °C under constant illumination provided by cool white fluorescent tubes at an irradiance of about 40 μmol m^{-2} s^{-1} incident on the surface of the growth vessels. Cells were harvested during the stationary phase by centrifugation (4000*g*, 20 min), lyophilized and stored at −20 °C until required.

Extraction of fatty acids

Cells of each sample of cyanobacteria (50–130 mg) were added to 50 mL of an MeOH–H_2O–HCl (30:3:1, v/v/v) mixture and held at 55 °C for 6 h. After cooling to 10 °C, 150 mL of cold water-*n*-pentane (2:1, v/v) mixture was added. The mixture was stirred for 1 h and then filtered through Whatman No. 1 filter paper. The pentane layer was concentrated to dryness under vacuum at 10 °C. The residue was extracted three times with 50 mL of CH_2Cl_2, filtered and concentrated to dryness under vacuum at 10 °C. The residues of pentane and CH_2Cl_2 extracts were combined and dissolved in 1.5 mL of cold pentane–CH_2Cl_2 (1:1, v/v) mixture, and obtained methyl ester of fatty and carboxylic acids used for GC-MS analysis. Standard carboxylic (C_4–C_9), dioic (C_4–C_9) and fatty (C_{12}–C_{18}) acids were obtained from ChemTech, Ltd. (Israel), esterified to methyl esters, and used for GC-MS analysis.

Gas chromatographic-mass spectrometric analysis

A Hewlett Packard 6890 (series II) gas chromatograph modified for glass-capillary work and a HP-GC mass selective detector (5973B MSD) were used. Fatty acid methyl esters were prepared according to Metcalfe and Wang (1981) and analyzed by GC on serially coupled capillary columns (Dembitsky et al., 1999): the RTX 1 column (30 m, ID 0.32 mm, film thickness 0.25 μm; Restek, USA) was coupled with a second capillary column (RTX 1701, 30 m, 0.32 mm, 0.25 μm film; Restek, USA). The GC oven was programmed for 40 °C per 2 min, increase 2 °C min^{-1} to 300 °C, 20 min at 300 °C. The injector temperature was kept at 180 °C. The flow rate of the carrier gas (helium) was 25 mL s^{-1}. The MS detector operated at 194 °C, ionization energy was 70 eV. The scan range was from 30 to 700 m/z at 0.9 scan per second. Solvent delay was 9 min. Fatty acid methyl esters were identified using mass spectral libraries search (Wiley 7th, and NIST-98).

Results and discussion

Biodiversity of fatty acids of the genus *Nostoc*

The six strains used in the present study (see Table 1) were identified based on morphological observations, belonging to terrestrial type: *Nostoc commune* Vauch. and *N. paludosum* Kütz; freshwater type: *N. pruniforme* Ag. and *N.verrucosum* Vauch.; and two symbiont species: *Nostoc* sp. isolated from lichen *Collema cristatum* and *Nostoc* sp. from lichen *Peltigera hydrothyria*, respectively. The composition and content of fatty acids in these strains are shown in Tables 2 and 3. Most of the fatty acids were monounsaturated (vary in different species from 7.7% to 51.5%, Table 3), di-, tri, and further polyunsaturated (16.3–56.8%) but *n*-saturated fatty acids (16.9–51.8%) were also identified with 16:0 as the most abundant. All the species of cyanobacteria appear to synthesize short (C3–C10) carboxylic acids, whose content is usually smaller than that of long-chain fatty acids (C14–C18). The major fatty acids were 16:0 ranging from 14.9% to 50.8% (of total fatty acids), (*Z*)-9-18:1, (*Z*,*Z*)-9,12-18:2, and also (*Z*,*Z*,*Z*)-9,12,15-18:3. The predominant unsaturation (39% of the total mono-unsaturated fatty acids) was at (*Z*)-9-18:1 (up to 19.8%). Other monounsaturation was observed at (*Z*)-9-16:1 and 11-16:1 (e.g., (*Z*)-9-hexadecenoic and 11-hexadecenoic acids). The principal fatty acids in the investigated cyanobacteria (Table 3) did not essentially differ from those of most previously studied cyanobacteria (Schneider et al., 1970; Murata and Nishida, 1987), but later found in some species (up to 25.7%).

Hydroxy (very from 0.4% to 0.7%) and dioic fatty acids (1.4–3.7%) were found as minor components. These acids were previously isolated by GC-MS from freshwater cyanobacteria belonging to the genus *Aphanizomenon* (Dembitsky et al., 2001) and *Nostoc* sp. (Dembitsky et al., 1999). Branched saturated fatty acids that were identified accounted for 1.1–8.6%. The fatty acid composition of cyanobacteria was studied by Holton et al. (1968) in *Anacystis nidulans* and by Lennarz (1966) in *Anabaena variabilis*. Major fatty acids thus far known to be present in cyanobacteria are hexadecanoic (16:0), 9-hexadecenoic (16:1), hexadecadienoic (16:2), octadecanoic (18:0), and 9-octadecenoic (18:1). Aliphatic dicarboxylic acids surprisingly afforded potent cytotoxicity and antineoplastic activity (Hall et al., 1999), and could serve as lipidoic markers for identification of some human and animal diseases (Martin, 1998; Ma et al., 1999). These acids are of major interest for medical specialists and biochemists.

Representatives of the genus *Nostoc* collected in various regions of the world have been investigated regarding their content of various fatty acids (see Tables 2 and 3); the members of the genus investigated are presented in Table 1. Data of the quantitative analysis of fatty acids are shown in Table 2, and in essence do not differ from published data. Special attention was given to dioic acids. The analysis of published data on *Nostoc* has shown that only Rezanka et al. (2003) have found some dioic acid in *Nostoc linkia*. With the usual columns, detection of dioic acids is rather problematic. However, in our experience use of coupled columns solves the problem perfectly (see Fig. 1). Doubtlessly, the greatest interest was taken in the composition of the saturated fatty acids (Table 2). The system used allows the separation and identification of the highly polar fatty acids.

The composition of monoenoic acids is shown in Table 3. Again, we came to the conclusion that our system enables the identification of considerably more monoenoic acids (5,7,9- and 11-isomers, for monoenoic acids) in the total lipid extract. It is possible to separate *trans*- and *cis*-isomers of various fatty acids. Polyenoic fatty acids (Table 3) present in various species of *Nostoc* are represented by a large spectrum of acids. It is difficult to interpret the absence of polyenoic acid in some species indicated by numbers 3,6,9-18:3 (26 and 29%, in *Nostoc* sp. 152 and *Nostoc* sp. PCC 73102,

Table 1. The *Nostoc* species investigated for their content of fatty acids

	Origin, place	Habitat	Reference
Phylum	Cyanobacteria		
Order	Nostocales		
Family	Nostocaceae		
Genus	*Nostoc*		
Species & strains			
1. *commune* UTEX 584	Gulf of Eilat, Israel	Marine	Potts et al. (1987)
2. *commune* UTEX 584	Texas University, collection	Freshwater	Olie and Potts (1986)
3. *commune* FACHB261	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
4. *commune* CCAP1453/2	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
5. *commune*	Gottingen University, collection	Freshwater	Vargas et al. (1998)
6. *commune*	Negev Desert, Israel	Terrestrial	This study
7. *ellipsosporum* CCAP1453/2	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
8. *ellipsosporum* CCAP1453/11	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
9. *ellipsosporum* CCAP1453/15	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
10. *ellipsosporum* CCAP1453/16	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
11. *ellipsosporum* CCAP1453/17	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
12. *ellipsosporum* CCAP1453/18	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
13. *ellipsosporum* CCAP1453/19	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
14. *flagelliforme*	Natural colony, China	Terrestrial	Liu et al. (2005)
15. *flagelliforme* FACHB838	Hong Kong University, collection	Terrestrial	Liu et al. (2005)
16. *flagelliforme* CCAP1453/33	Hong Kong University, collection	Terrestrial	Liu et al. (2005)
17. *linckia*	Ein Boqeq Springs, Israel	Freshwater	Rezanka et al. (2003)
18. *muscorum* UTEX389	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
19. *muscorum*	Texas University, collection	Freshwater	Parker et al. (1967)
20. *muscorum*	Tennessee University, collection	Freshwater	Holton et al. (1968)
21. *paludosum*	Lagoon Albufera de Valencia, Spain	Marine	Vargas et al., (1998)
22. *paludosum*	Dead Sea area, Israel	Terrestrial	This study
23. *pruniforme*	Lake Kinneret, Israel	Freshwater	This study
24. *punctiforme* UTEX1833	Hong Kong Univesity, collection	Terrestrial	Liu et al. (2003)
25. *punctiforme*	University of Tokyo, collection	Freshwater	Watanabe and Yamamoto (1968)
26. *verrucosum*	Hula Lake, Israel	Freshwater	This study
27. sp.	*Collema* sp., lichen	Photobiont	Dembitsky et al. (1999)
28. sp.	*Collema cristatum*, lichen	Photobiont	This study
29. sp.	*Peltigera horizontalis*, lichen	Photobiont	Guschina et al. (2003)
30. sp.	*Peltigera hydrothyria*, lichen	Photobiont	This study
31. sp.	*Azolla caroliniana*, fern	Photobiont	Caudales et al. (1993)
32. sp. ANT	Ross Ice Shelf, Antarctica	Marine	Potts et al. (1987)
33. sp. Albufera	Lagoon Albufera de Valencia, Spain	Marine	Vargas et al., (1998)
34. sp. Caquena	Lake, Chile	Freshwater	Vargas et al., (1998)
35. sp. Chile	Lake, Chile	Freshwater	Vargas et al., (1998)
36. sp. Chucula	Caquena River, Chile	Freshwater	Vargas et al., (1998)
37. sp. FACHB713	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
38. sp. FACHB599	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
39. sp. HUN	Hunan Provice, China	Terrestrial	Potts et al. (1987)
40. sp. Llaita	Caquena River, Chile	Freshwater	Vargas et al. (1998)
41. sp. Loa	Loa River, Chile	Freshwater	Vargas et al. (1998)
42. sp. REICH	Reichenau, Constance, Germany	Terrestrial	Potts et al. (1987)
43. sp. UTEX1544	Hong Kong University, collection	Terrestrial	Liu et al. (2003)
44. sp. ATCC 27895	Rutgers University	Freshwater	Caudales and Wells (1992)
45. sp.	Houston University, collection	Terrestrial	Schneider et al. (1970)
46. sp. 152	Lake Saaskjarvi, Finland	Freshwater	Gugger et al. (2002)
47. sp. PCC73102	Root section, Australia	Terrestrial	Gugger et al. (2002)

see Table 3). It is possible, that these are artifacts. Fatty acid biosynthesis in some cyanobacterial species has recently been reviewed (Murata and Siegenthaler, 1998). In cyanobacterial cells, fatty acids are synthesized via a mechanism similar to that in the cells of higher plants (Murata and

Table 2. Comparison of the proportion of hydroxy, dioic and saturated fatty acids obtained in the present study with data reported in literature for *Nostoc* species

Acid type (systematic name)	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Hydroxy fatty acids																	**0.7**							
2-Hydroxypropanoic (2-OH-3:0)																	0.2							
3-Hydroxybutanoic (3-OH-4:0)																	0.2							
2-Hydroxy-4-methylpentanoic (2-OH-4-Me-5:0)																	0.1						0.1	
2-Hydroxy-3-methylpentanoic (2-OH-3-Me-5:0)																	0.2						0.1	
Dioic fatty acids						**1.4**											**3.7**					**2.4**	**3.2**	
Propane-1,3-dioic																							0.1	
Butane-1,4-dioic																	0.4						0.3	
2-OH-Butane-1,4-dioic																						0.4		
Pentane-1,5-dioic						0.1											0.4						0.4	
Hexane-1,6-dioic						0.9											0.3					1.4	1.1	
Heptane-1,7-dioic						0.3											1.5						0.5	
Octane-1,8-dioic						0.1											0.3					0.6	0.4	
Nonane-1,9-dioic																	0.8						0.4	
n-Saturated fatty acids	**33.6**	**35.6**	**30.6**	**29.6**	**41.2**	**30.0**	**28.9**	**31.2**	**29.6**	**31.4**	**31.2**	**30.6**	**27.7**	**24.2**	**28.1**	**26.9**	**40.6**	**25.9**	**35.0**	**50.5**	**36.8**	**29.2**	**27.8**	**26.1**
Butanoic (4:0)																	0.1							
Pentanoic (5:0)						0.1											0.1							
Hexanoic (6:0)						0.2											0.1						0.5	
Heptanoic (7:0)																	0.1						0.2	
Octanoic (8:0)						0.3											0.2					0.7	0.4	
Nonanoic (9:0)						0.1											0.1						0.2	
Decanoic (10:0)						0.5											0.2					0.5	0.6	
Undecanoic (11:0)						0.4											0.1							
Dodecanoic (12:0)						0.9											0.3			1.0		0.8	0.6	
Tridecanoic (13:0)						0.6											0.3					0.3	0.3	
Tetradecanoic (14:0)			0.5	1.2	0.3	2.4	2.3	2.8	6.7	5.3	2.8	2.2	1.3	0.1	0.6	0.9	1.3	0.9	2.7	16.0	0.4	2.1	1.3	2.0
Pentadecanoic (15:0)						1.2											1.1		2.2			0.4	0.7	
Hexadecanoic (16:0)	33.2	23.1	27.8	23.5	39.5	20.7	19.3	21.6	17.0	19.4	23.8	19.5	19.1	23.5	21.3	17.9	33.0	16.7	27.0	31.5	35.4	21.7	19.8	14.9
Heptadecanoic (17:0)	0.4					0.7											0.8					0.5	0.5	
Octadecanoic (18:0)		9.5	2.3	4.9	1.4	1.9	7.3	6.8	5.9	6.9	4.6	7.8	7.3	0.6	6.2	8.1	2.5	8.3	3.1	2.0	1.0	2.2	1.5	7.2
Eicosanoic (20:0)		3.0																					1.2	
Branched saturated fatty acids	**8.6**					**4.1**											**6.1**					**1.1**	**2.8**	
2-Methylbutanoic (2-Me-4:0)																	0.1							
3-Methylbutanoic (3-Me-4:0)																	0.1							
4-Methylhexanoic (4-Me-6:0)						0.3											0.2							
8-Methyldecanoic (8-Me-10:0)						0.3											0.2							
11-Methyldodecanoic (11-Me-12:0)																	0.2						0.2	
10-Methyldodecanoic (10-Me-12:0)						0.4																		
12-Methyltridecanoic (12-Me-13:0)																	0.3							
11-Methyltetradecanoic (11-Me-14:0)						0.2																		
12-Methyltetradecanoic (12-Me-14:0)																	0.4					0.4		
13-Methyltetradecanoic (13-Me-14:0)	0.7					0.3											0.4						0.6	
14-Methylpentadecanoic (14-Me-15:0)	Tr					0.5											0.5					0.3	0.4	
2-Methylhexadecanoic (2-Me-16:0)																	0.6							
10-Methylhexadecanoic (10-Me-16:0)																								
14-Methylhexadecanoic (14-Me-16:0)						0.4											0.3						0.3	
15-Methylhexadecanoic (15-Me-16:0)	0.6					0.6											0.5					0.4		
10-Methylheptadecanoic (10-Me-17:0)																	0.5							
16-Methylheptadecanoic (16-Me-17:0)																	0.4						0.3	
15-Methylheptadecanoic (15-Me-17:0)																	0.5							
2-Hexylcyclopropaneoctanoic (Cyc-17:0)						0.6											0.9						0.4	
2-Hexylcyclopropanedecanoic (Cyc-19:0)	7.3					0.5																	0.6	

	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
Hydroxy fatty acids				**0.4**		**0.6**																	
2-Hydroxypropanoic (2-OH-3:0)				0.1																			
3-Hydroxybutanoic (3-OH-4:0)				0.1		0.1																	
2-Hydroxy-4-methylpentanoic (2-OH-4-Me-5:0)				0.1		0.2																	
2-Hydroxy-3-methylpentanoic (2-OH-3-Me-5:0)				0.1		0.3																	
Dioic fatty acids		**1.7**		**1.4**		**2.4**																	
Propane-1,3-dioic						0.4																	
Butane-1,4-dioic		0.2				0.1																	
2-OH-Butane-1,4-dioic		0.2		0.1		0.2																	
Pentane-1,5-dioic				0.2																			
Hexane-1,6-dioic		0.6		0.3		0.6																	
Heptane-1,7-dioic				0.2																			
Octane-1,8-dioic		0.7		0.4		0.4																	
Nonane-1,9-dioic				0.2		0.7																	
n-Saturated fatty acids	**29.9**	**32.8**	**67.5**	**26.1**	**40.2**	**31.8**	**40.7**	**33.7**	**51.8**	**36.0**	**44.3**	**32.2**	**22.6**	**26.2**	**27.1**	**32.4**	**31.5**	**31.2**	**27.2**	**24.8**	**41.6**	**22.0**	**16.9**
Butanoic (4:0)		0.2																					
Pentanoic (5:0)		0.1																					
Hexanoic (6:0)		0.2		0.3																			
Heptanoic (7:0)				0.2																			
Octanoic (8:0)		0.2		0.1		0.3																	
Nonanoic (9:0)				0.1		0.2																	
Decanoic (10:0)		0.2		0.2																			
Undecanoic (11:0)				0.3																			
Dodecanoic (12:0)		0.6				0.5																	
Tridecanoic (13:0)		0.3		0.2		0.3																	
Tetradecanoic (14:0)		2.8	18.3	1.8	0.7	2.5	2.1		0.3	0.4	0.3	0.3	2.1	1.0		0.5	0.4		2.3	0.3	2.3	3.4	tr
Pentadecanoic (15:0)		1.1	10.2	0.4		0.4																	
Hexadecanoic (16:0)	28.8	23.7	26.7	19.6	37.4	24.8	36.7	32.0	50.8	34.2	43.1	30.9	14.4	16.9	23.1	30.7	30.1	30.4	16.7	23.2	36.3	18.4	16.0
Heptadecanoic (17:0)		0.2		0.3		0.2		0.2							0.4			0.1					
Octadecanoic (18:0)	1.1	3.2	12.3	2.6	2.1	2.6	1.9	1.5	0.7	1.4	0.9	1.0	6.1	8.3	3.6	1.2	1.0	0.7	8.2	1.3	3.0	tr	tr
Eicosanoic (20:0)																							
Branched saturated fatty acids		**0.9**		**1.0**		**1.4**	**1.9**								**1.1**			**1.4**		**3.6**		**4.1**	**1.2**
2-Methylbutanoic (2-Me-4:0)																							
3-Methylbutanoic (3-Me-4:0)				0.1																			
4-Methylhexanoic (4-Me-6:0)						0.2																	
8-Methyldecanoic (8-Me-10:0)				0.3																			
11-Methyldodecanoic (11-Me-12:0)																							
10-Methyldodecanoic (10-Me-12:0)																							1.2
12-Methyltridecanoic (12-Me-13:0)																							
11-Methyltetradecanoic (11-Me-14:0)																							
12-Methyltetradecanoic (12-Me-14:0)																							
13-Methyltetradecanoic (13-Me-14:0)				0.2											0.5			0.4					
14-Methylpentadecanoic (14-Me-15:0)		0.4				0.3									1.0			0.4		2.1			
2-Methylhexadecanoic (2-Me-16:0)																							
10-Methylhexadecanoic (10-Me-16:0)																						2.3	tr
14-Methylhexadecanoic (14-Me-16:0)																							
15-Methylhexadecanoic (15-Me-16:0)		0.5				0.6	1.9								0.6			0.6		1.4			
10-Methylheptadecanoic (10-Me-17:0)																						1.8	
16-Methylheptadecanoic (16-Me-17:0)				0.4																			
15-Methylheptadecanoic (15-Me-17:0)																							
2-Hexylcyclopropaneoctanoic (Cyc-17:0)																							
2-Hexylcyclopropanedecanoic (Cyc-19:0)						0.3									tr					0.1			

*Amount of fatty acids gave in percentage of total fatty acids.

Table 3. Comparison of the proportion of fatty acids obtained in the present study with data reported in literature for *Nostoc* species

Acid type (systematic name)	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Monounsaturated fatty acids	**28.7**	**7.7**	**39.3**	**40.3**	**16.6**	**28.0**	**38.9**	**34.8**	**38.4**	**36.4**	**36.5**	**39.7**	**42.4**	**33.1**	**37.5**	**39.4**	**24.5**	**49.7**	**38.1**	**30.4**	**31.2**	**27.2**	**17.3**	**51.1**
C12:1																								
C14:1 (all isomers)						0.3													2.1	2.0				
7-Tetradecenoic (7-14:1)																								
9-Tetradecenoic (9-14:1)																								
14-Methylpentadecenoic (14-Me-11-15:1)	tr																							
C16:1 (all isomers)			25.5	24.4			22.0	18.9	22.5	21.9	19.2	25.8	24.5	29.4	14.9	17.3		27.9	20.0	15.0				27.0
5-Hexadecenoic (5-16:1)	tr					2.9																1.4	0.7	
7-Hexadecenoic (7-16:1)	13.5				10.3	3.6															18.0	1.8	1.1	
9-Hexadecenoic (9-16:1)	tr					5.1											7.1					6.4	3.2	
11-Hexadecenoic (11-16:1)						0.6											2.1					0.9	0.4	
trans-9-16:1																								
C17:1	2.6					0.4														6.0		0.5	0.3	
C18:1 (all isomers)			13.8	15.9			16.9	15.9	15.9	14.5	17.3	13.9	17.9	3.7	22.6	22.1		21.8	16.0	7.4				24.1
5-Octadecenoic (5-18:1)						1.3																0.9	0.5	
7-Octadecenoic (7-18:1)	8.5				1.1	2.6															1.3	2.1	3.2	
9-Octadecenoic (9-18:1)	4.1	7.7			5.2	11.2											15.3				11.9	13.3	7.9	
br-18:1	tr																					0.3		
br-19:1	tr																							
trans-9-18:1	tr																							
Unsaturated fatty acids	**21.1**	**56.8**	**30.0**	**30.8**	**42.4**	**36.5**	**32.2**	**33.9**	**31.9**	**31.9**	**32.3**	**30.8**	**29.8**	**43.1**	**34.3**	**33.7**	**17.8**	**25.4**	**25.0**	**31.2**	**31.9**	**39.8**	**48.9**	**24.7**
C16:2 (all isomers)																								
6,9-Headecadienoic (16:2)														8.7	15.0	14.9	1.4				0.2	3.8		
7,10-Hexadecadienoic (16:2)					0.4	2.1													14.0	10.1			2.8	
4,7,10-16:3																								
C16:4n-1					0.4																	0.2		
All isomers (18:2)																								
9,12-Octadecadienoic (18:2)	17.8		15.2	16.0	17.6	14.5	14.0	12.6	11.0	12.4	12.9	15.8	12.9				11.2	14.1			19.1	15.1	17.2	13.5
10,13-Octadecadienoic (18:2)						2.1											1.9					3.1	4.2	
12,15-Octadecenoic (18:2)						1.1											0.9					0.6	0.9	
All isomers (18:3)																			11.0	21.1				
9,12,15-Octadecatrienoic	3.3		14.8	14.8		13.5	18.2	21.3	20.9	19.5	19.4	15.0	16.9	34.4	19.3	18.8	2.4	11.3				15.3	16.9	11.2
6,9,12-Octadecatrienoic					24.0	3.2															12.4	1.8	5.8	
3,6,9-Octadecatrienoic (18:3)																								
8,11,14-Eicosatrienoic (20:3)		56.8																						
All isomers (18:4)																								
6,9,12,15-Octadecatetraenoic (18:4)																						1.1		
C20:5																								

	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
Monounsaturated fatty acids	**28.6**	**15.2**	**10.3**	**17.5**	**29.6**	**20.1**	**25.0**	**42.4**	**6.1**	**40.5**	**19.2**	**32.0**	**46.7**	**45.8**	**43.3**	**31.6**	**35.0**	**29.5**	**44.7**	**31.6**	**29.8**	**21.2**	**25.7**
C12:1							2.0																
C14:1 (all isomers)							2.9								0.6								
7-Tetradecenoic (7-14:1)																				0.1			
9-Tetradecenoic (9-14:1)																				3.4			
14-Methylpentadecenoic (14-Me-11-15:1)															0.6					2.1			
C16:1 (all isomers)	18.4												28.1	24.9	10.1				25.6		10.0		
5-Hexadecenoic (5-16:1)		0.6		0.9		1.2		1.2							1.2			0.7				10.4	
7-Hexadecenoic (7-16:1)		2.2		3.1		2.7	4.3	18.3	3.3	20.9	8.8	19.5				20.2	21.9	13.0		0.4		10.8	25.7
9-Hexadecenoic (9-16:1)		1.9		1.8	16.8	2.3		0.4										0.1		18.7		tr	
11-Hexadecenoic (11-16:1)		0.4		1.1		1.3														0.4			

trans-9-16:1							0.3												1.4			
C17:1		0.2		0.4		0.6		0.8							1.3			0.6				
C18:1 (all isomers)	10.2												18.6	20.9					19.1			
5-Octadecenoic (5-18:1)		0.7		1.1		1.4																
7-Octadecenoic (7-18:1)		2.3		5.2		6.1		4.4	0.2	1.7	0.8	2.5			20.6	1.9	1.6	4.2				
9-Octadecenoic (9-18:1)		6.9	10.3	3.9	12.8	4.3	15.5	16.2	2.6	17.9	9.6	10.0			7.8	9.5	11.5	10.4		5.1	19.8	
br-18:1							0.4	1.1							1.1			0.6				
br-19:1															tr							
trans-9-18:1																				1.9		
Unsaturated fatty acids	**42.7**	**47.9**	**16.3**	**53.6**	**29.0**	**43.5**	**44.1**	**25.0**	**42.1**	**23.5**	**36.6**	**35.7**	**30.7**	**27.9**	**27.5**	**36.0**	**33.6**	**37.5**	**28.1**	**32.1**	**15.5**	**46.0**	**46.7**
C16:2 (all isomers)	24.5																				1.0		
6,9-Headecadienoic (16:2)																						20.5	17.8
7,10-Hexadecadienoic (16:2)		2.2	7.5	3.1	0.7	2.6	3.5	4.5	0.3	0.3	0.2	0.3			15.6	0.4	0.4	9.3				
4,7,10-16:3																				1.5		
C16:4n-1				1.2		0.8			0.1				0.3			0.2	1.3			0.9		
All isomers (18:2)																						
9,12-Octadecadienoic (18:2)		18.6		19.1	14.4	15.3	18.1	4.5	6.5	23.2	15.7	19.3	14.0	13.0	11.9	15.9	15.5	28.2	14.1	12.4	8.0		
10,13-Octadecadienoic (18:2)		3.1		0.3		0.4																	
12,15-Octadecenoic (18:2)		0.4		0.3		0.7																	
All isomers (18:3)																					6.5		
9,12,15-Octadecatrienoic	18.2	19.4	8.8	18.1	13.9	17.4	21.9	16.0	3.2		18.0	15.8	16.7	14.9		19.5	16.4		14.0	17.3			
6,9,12-Octadecatrienoic		4.2		9.2		4.2			11.6		0.5											tr	tr
3,6,9-Octadecatrienoic (18:3)																						25.5	28.9
8,11,14-Eicosatrienoic (20:3)																							
All isomers (18:4)											1.9												
6,9,12,15-Octadecatetraenoic (18:4)				2.3		2.1			19.0		0.3												
C20:5								0.6	1.4														

*Amount of fatty acids gave in percentage of total fatty acids.

Nishida, 1987; Nishida and Murata, 1996). 16:0-ACP and 18:0-ACP are synthesized as end-products by the fatty acid synthase system (Stapleton and Jaworski, 1984). In cyanobacteria, the biosynthesis of unsaturated fatty acids is initiated by Δ9 acyl-lipid desaturase which introduces the first double bond at the Δ9 position of a saturated fatty acid that has been esterified to a glycerolipid (Sato, 1993; Sakamoto et al., 1994). Obtained results indicate that the Δ9 acyl-lipid desaturases are specific to stearic acid esterified at the C-1 position of a glycerolipid and are nonspecific with respect to the polar head group of the glycerolipid. Neither acyl-ACP desaturase nor acyl-ACP hydrolase has been found in cyanobacterial cells. The acyl groups of acyl-ACPs are directly incorporated into glycerolipids by acyltransferases and then converted to unsaturated fatty acids by acyl-lipid desaturases. Thus, the levels of unsaturated fatty acids in the membrane lipids of cyanobacterial cells are determined exclusively by the acyl-lipid desaturases (Murata and Nishida, 1987).

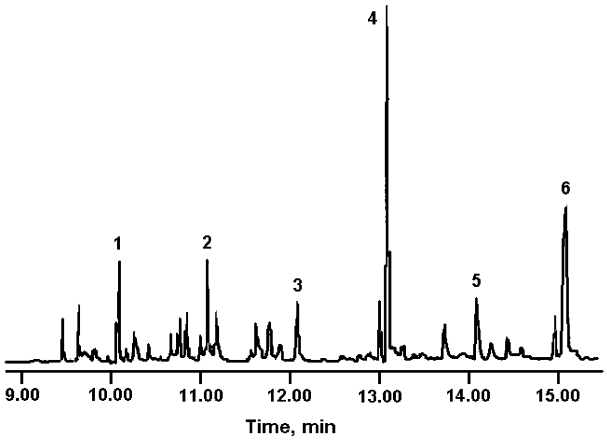

Figure 1. The part of GC chromatogram (full run is 162 min) separation on serially coupled capillary columns of some low-molecular dimethyl esters of dioic acids identified from cultured culture of *Nostoc* sp., isolated from lichen *Collema cristatum*. Compounds associated with the numbered peaks: 1, butane-1,4-dioic; 2, pentane-1,5-dioic; 3, hexane-1,6-dioic; 4, heptane-1,7-dioic; 5, octane-1,8-dioic acid; 6, nonane-1,9-dioic. Amounts of indicated dioic acids see Table 2.

It is known that particular groups of organisms are characterized by particular fatty acids (Kerwin, 1994) that can be used as their biological markers. Our chromatographic assay allowed us to discover previously unknown acids, such as, e.g., methyl-branched-saturated and dioic acids (Table 2), in some freshwater wild cyanobacteria and showed

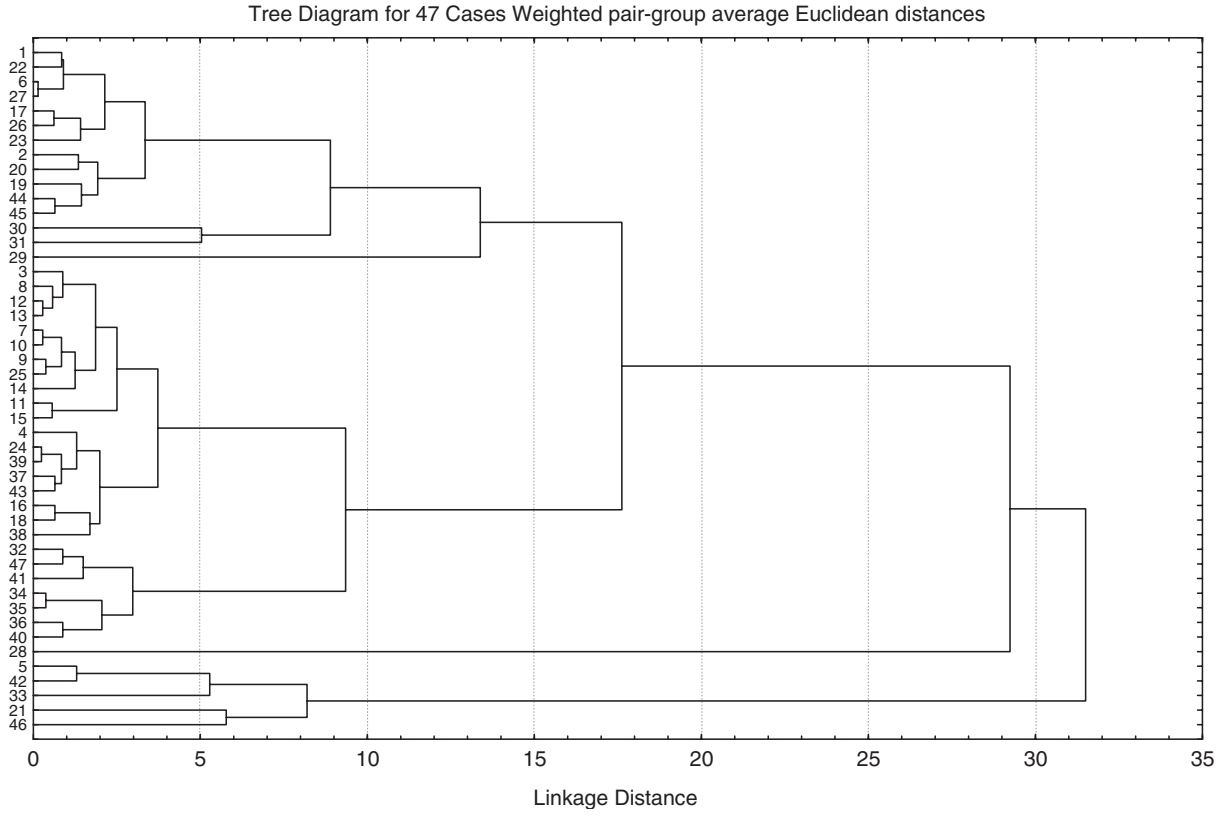

Figure 2. Dendrogram obtained by hierarchical cluster analysis of 47 *Nostoc* species.

the presence of more than 50 fatty acids in some cyanobacterial species.

Fatty acids of cyanobacteria have been investigated for their content, composition types, metabolism, and biotechnological application by different researchers (Ahlgren et al., 1992; Murata et al., 1992; Vargas et al., 1998; Li and Liu, 2003). In terms of chemotaxonomy by fatty acid composition, Li and Watanabe (2001) indicated that there should be a separation between 24 axenic strains of planktonic *Anabaena* with straight trichomes, in the genus *Anabaena* based on the difference in their fatty acid composition. Caudales and Wells (1992) as well as Kruger et al. (1995) also demonstrate the importance of fatty acid composition in the taxonomy of cyanobacteria at the genus and subgenus levels. Our previous study shows that hydrocarbon and fatty acid composition can be used in chemotaxonomy of cyanobacteria even at the species level based on examination of 55 strains of cyanobacteria (Dembitsky and Srebnik, 2002; Rezanka et al., 2003; Dembitsky and Rezanka, 2005). In the present study, fatty acids and their chemo-taxonomic values were further investigated using more strains of *Nostoc*. The results of fatty acid analysis of *Nostoc* species comprise with literature data show in Tables 2 and 3.

From literature data, it can be concluded that some filamentous nitrogen-fixing cyanobacteria represent a source of essential fatty acids that are of commercial interest, including linoleic, and α- and γ-linolenic acids, among others. The presence of γ-linolenic acid in *Nostoc* sp. (Chile) and *Anabaenopsis* sp. is an interesting finding, because this fatty acid has not previously been reported to be present in strains belonging to these genera (Vargas et al., 1998).

Investigation of different *Nostoc* species collected from marine, freshwater, terrestrial ecosystems, and also in symbiotic species shown a large biodiversity of fatty acids (more than 80 fatty acids).

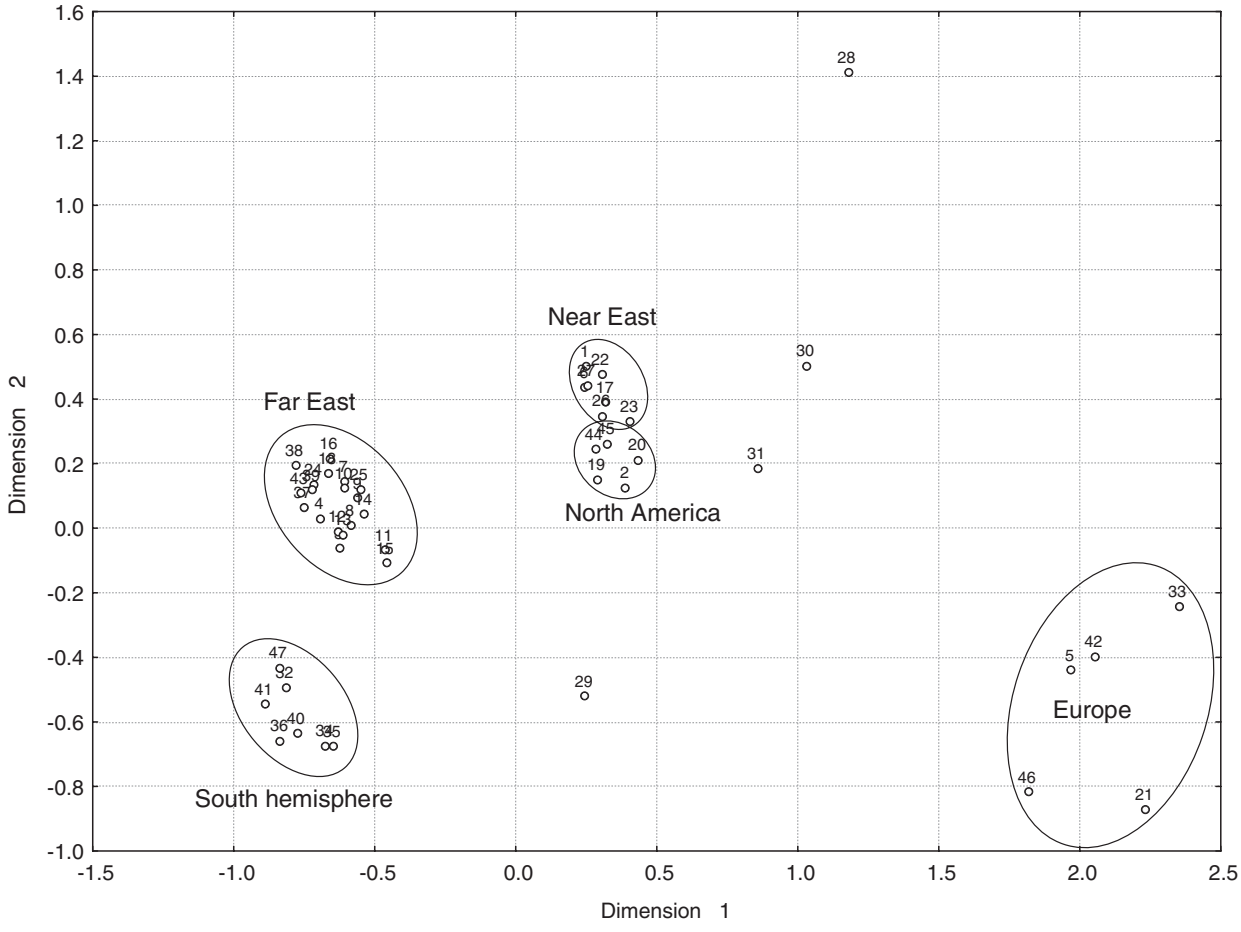

Figure 3. Two-dimensional graph of a final configuration from the multidimensional scaling of 47 species of *Nostoc*. Circles correspond to some clusters in Fig. 2 (some of them correspond to geographical places).

Statistic analysis and chemotaxonomy of the *Nostoc* species

The input data for cluster analysis are shown in Tables 1 and 2. As the distance measure in hierarchical cluster analysis, Euclidean distance measure was used. It is the geometric distance in multidimensional space. For two objects i and i' it is computed as

$$d_{ii'} = \sqrt{\sum_{j=1}^{p}(x_{ij} - x_{i'j})^2},$$

where p is number of variables and x_{ij} and $x_{i'j}$ are individual values in the data matrix. The values are in the same units, so a standardization of values has no influence on the results of analysis. Squared Euclidean distance measure was not used by reason of a less number of clusters (there are smaller differences between objects).

As the linkage rules, four methods were applied: weighted pair-group average, unweighted pair-group average, single linkage (nearest neighbor) and complete linkage (furthest neighbor). They are chosen as some other methods can be used only with squared Euclidean distance measure. By the weighted pair-group average method (WPGMA), we obtained the dendrogram (see Fig. 2) which is the most illustrative from those obtained by all mentioned methods. In this method, the distance between two clusters is calculated as the average distance between all possible pairs of objects from the two distinct clusters. The size of the respective clusters (i.e. the number of objects contained in them) is used as a weight.

Figure 2 shows results obtained by cluster analysis where individual clusters are clearly separated. From these results, it is evident that the place from which a sample was acquired is more important than taxonomical affinity. We were succeeded in the definition of five clusters based on geographical origin, i.e. Europe, Near East, Far East, North America, and South hemisphere (Australia, Antarctica, and South America). On the contrary, we were not succeeding in assignment of samples from numbers 28 to 31. It is necessary to say that all four species were isolated as photobionts from lichens or fern (it is the possibility to itemize this topic).

The separation of individual clusters is very illustrative, which is seen better in Fig. 3 than in Fig. 2. For example, the cluster of different species of *Nostoc* denoted as South Hemisphere includes samples collected in Chile, Australia, and Antarctica. In this cluster, a more detailed separation can be performed. Therefore, species No. 36 and 40 (from Caquena River, Chile) create a pair clearly

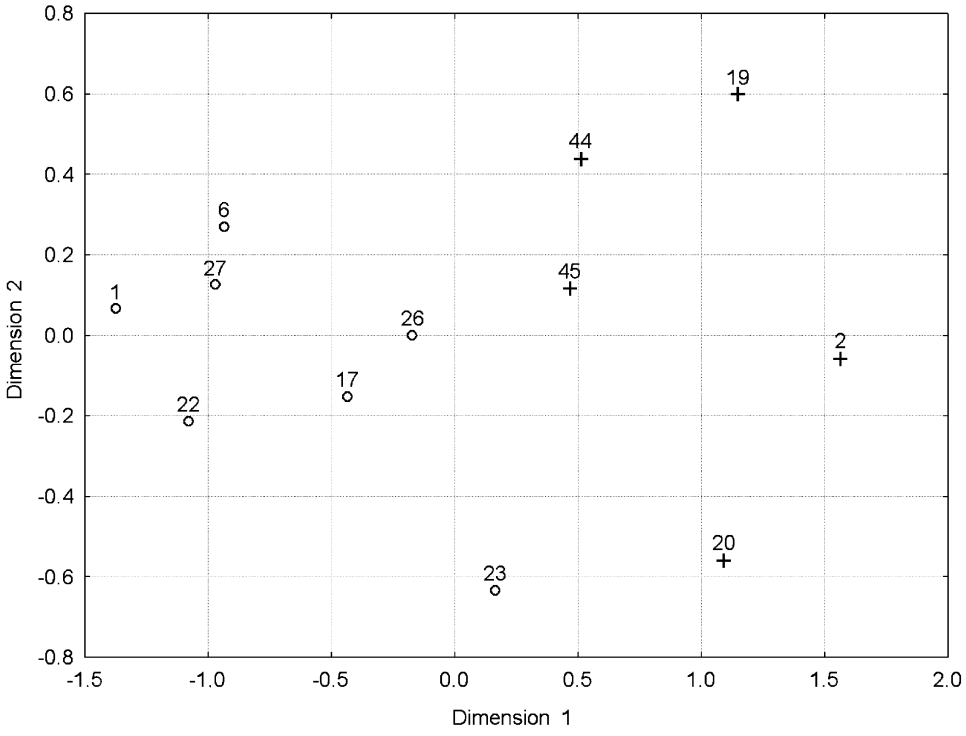

Figure 4. Two-dimensional graph of the final configuration from a multidimensional scaling of Near East (circle symbol) and North America (cross symbol) species of *Nostoc* species.

separated from other species. In addition, the species No. 34 and 35 from Lake, Chile create second pair. Remaining three species, i.e. No. 32 from Ross Ice Shelf, Antarctica, No. 41 from Loa River, Chile and No. 47 from Australia are also clearly separated.

For close clusters of blue-green alga *Nostoc* collected at Near East and North America, we used more detailed separation by selection of only these species for the analysis. From two-dimensional graph in Fig. 4, it can be seen the clear separation of both clusters. At three-dimensional space shown in Fig. 5, the separation of these clusters is visible yet more. Again, we could describe distances of individual points in more details. We can mention the separation of different species of *Nostoc* collected in Israel as an example. *Nostoc commune* (No. 1) from sea is clearly separated of the others. In the case of the further species, the situation is similar. For example, three different freshwater strains, i.e. *N. linckia* (No. 17) from Ein Boqeq Springs, *N. verrucosum* (No. 26) from Hula Lake, and *N. pruniforme* (No. 23) from Lake Kinneret, differ from samples of *Nostoc* collected in arid area, sea or as photobiont, see Fig. 2. However, we were not succeeded in collection of two different species of *Nostoc* in any locality (again, It is the possibility to itemize this topic.).

All samples exhibited the characteristic composition of their original populations, indicating that the fatty acids composition of different strains of *Nostoc* is under environmental control rather than genetic control.

On the contrary, in the paper (Khotimchenko et al., 2002) it is mentioned that a comparison of the fatty acid compositions of red, brown and green algae from different regions of the world and from the coast of northern California showed that, in general, and independently of the geographical source, algae had similar FA profiles typical of each phylum of seaweeds.

Viso and Marty (1993) were studied fatty acid composition and C/N ratios of 28 marine microalgae from nine taxonomic classes. A combination of several criteria was selected to discriminate taxonomic classes: 16:4(n-1), 20:5(n-3), 22:6(n-3), C18 polyunsaturated fatty acids (PUFA), C20PUFA, C22PUFA, 16:1(n-7)/16:0, C16FA/C18FA, C16PUFA/C18PUFA, 18:5(n-3)/18:3(n-3). Obtained results expressed in terms of relative abundance (percent of total fatty acids) and absolute abundance (ng FA mg^{-1} C and pg FA $cell^{-1}$) led to different interpretations concerning the nutritional value of the different species as food for organisms in culture or in the natural environment.

This research has shown that in order to identify and authenticate different strains of *Nostoc* with certainty using chemical profiling, it is necessary to study the profiles of as many strains as possible, collected from different areas of the World.

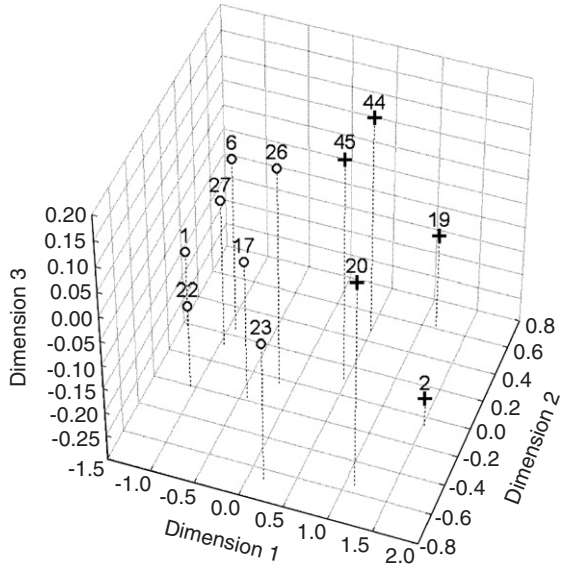

Figure 5. Three-dimensional graph of a final configuration from the multidimensional scaling of Near East (circle symbol) and North America (cross symbol) of *Nostoc* species.

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